A NOVEL METHOD OF DIAGNOSING, MONITORING, STAGING, IMAGING AND TREATING GYNECOLOGIC CANCERS

FIELD OF THE INVENTION

This invention relates, in part, to newly developed assays for detecting, diagnosing, monitoring, staging, prognosticating, imaging and treating cancers, particularly gynecologic cancers including endometrial, mammary, ovary and uterine cancer.

BACKGROUND OF THE INVENTION

In women, gynecologic cancers account for more than one-fourth of the malignancies.

For example, endometrial cancer occurs at a rate of approximately 44,500 new cases per year with approximately 10,000 deaths per year. If diagnosed and treated early, when the cancer is still confined to the endometrium, cure can be achieved in approximately 95% of the cases by hysterectomy. Pap smears can show endometrial cancers but are effective in only 50% of the cases. For the remainder, abnormal vaginal bleeding is typically the first clinical sign of endometrial cancer.

Sarcoma of the uterus, a very rare kind of cancer in women, is a disease in which cancer (malignant) cells start growing in the muscles or other supporting tissues of the uterus. Sarcoma of the uterus is different from cancer of the endometrium, a disease in which cancer cells start growing in the lining of the uterus. Women who have received therapy with high-dose x-rays (external beam radiation therapy) to their pelvis are at a higher risk to develop sarcoma of the uterus. These x-rays are sometimes given to women to stop bleeding from the uterus. Like most cancers, sarcoma of the uterus is best treated when it is found (diagnosed) early. Sarcoma of the uterus usually begins after menopause. When

a patient has signs of such cancer, an internal pelvic examination is usually performed to detect any lumps or changes in shape of the pelvic organs. A Pap test may also be performed, however because sarcoma of the uterus begins inside the organ, this cancer is not usually detected by the Pap test. A dilation and curettage (D&C) may also be performed and a biopsy sample taken and examined microscopically.

It is estimated that one of every nine women in America will develop breast cancer sometime during her life based on a lifespan of 85 years. Annually, over 180,000 women in the United States are diagnosed with breast cancer and approximately 46,000 die from this disease. Every woman is at risk for breast cancer. However, a woman's chances of developing breast cancer increase as she grows older; 80 percent of all cancers are found in women over the age of 50. There are also several risk factors that can increase a woman's chances of developing breast cancer. These include a family history of breast cancer, having no children or the first child after the age of 30, and an early start of menstruation. However, more than 70 percent of women who develop breast cancer have no known risk factors. Less than 10 percent of breast cancer cases are thought to be related to the BRCA1 gene discovered in 1994. Researchers are now investigating the role of other factors such as nutrition, alcohol, exercise, smoking, and oral contraceptives in development of this gynecologic cancer. Mammograms, or special x-rays of the breast, can detect more than 90 percent of all cancers.

Carcinoma of the ovary is another very common gynecologic cancer. In fact, ovarian cancer causes more deaths than any other cancer of the female reproductive system. Approximately one in 70 women develop ovarian cancer during their lifetime. An estimated 14,500 deaths in 1995 resulted from ovarian cancer. Ovarian cancer often does not

cause any noticeable symptoms. Possible warning signals include an enlarged abdomen due to an accumulation of fluid or vague digestive disturbances (discomfort, gas or distention) in women over 40. In rare cases abnormal vaginal bleeding also occurs. Pap tests do not detect ovarian cancer. Thus, periodic, complete pelvic examinations are important and recommended annually for women over 40.

In all of these gynecologic cancers, chances of survival are much better if the cancer is diagnosed at an early stage. Further, treatment decisions for the individual are linked to the stage of the cancer present in that individual. However, current cancer staging methods are limited and some such cancers initially staged as not metastatic are actually metastatic. Discovery of metastasis is significant because patients with metastatic cancers have a poorer prognosis and require significantly different therapy than those with localized cancers.

Accordingly, there is a great need for sensitive and accurate methods for early detection and staging of gynecologic cancers such as endometrial, breast, uterine and ovarian cancer in a human to determine whether or not such cancer has metastasized and for monitoring the progress of such cancer in a human which has not metastasized for the onset of metastasis.

Steroid binding proteins, including uteroglobin and CC10, are a class of proteins which bind steroids along with methylsulfonyl metabolites of polychlorinated biphenyls. The exact function of members of this class of protein is uncertain. However, uteroglobin has been shown to inhibit PLA_2 mediated responses.

Gene and gene products homologous to uteroglobin are described in WO 97/34997 entitled Human Endometrial Specific Steroid Binding Factors I, II and III. The genes and their encoded products are referred to as Human Endometrial Specific Steroid-Binding Factors I, II and III (hESF I, II, and III).

Methods for utilizing these genes and gene products in research and diagnostic and clinical arts are disclosed. In particular, methods for detecting mutations in the hESFI, II or III gene or altered protein expression resulting from a mutant gene are indicated to be useful in diagnosing susceptibility to asthma and endometrial cancer.

A gene and gene product homologous to uteroglobin and very similar to hESF III, referred to as human mammoglobin homolog or HGH, is also described in WO 99/19487. The human mammoglobin homolog is suggested to be useful for the diagnosis, prevention and treatment of neoplastic disorders and endometriosis.

It has now been found that detection of hESF III, referred to herein as ESBPIII, is useful in diagnosing, monitoring, staging, prognosticating, imaging and treating cancers, particularly gynecologic cancers including endometrial, mammary, ovary and uterine cancer.

Accordingly, in the present invention, methods are provided for detecting, diagnosing, monitoring, staging, prognosticating, imaging and treating gynecologic cancers via ESBPIII. ESBPIII refers, among other things, to native protein expressed by the gene comprising the polynucleotide sequence of SEQ ID NO:1. The amino acid sequence of a polypeptide encoded by SEQ ID NO:1 is depicted herein as SEQ ID NO:2. In the alternative, what is meant by the ESBPIII as used herein, means the native mRNA encoded by the gene comprising the polynucleotide sequence of SEQ ID NO:1 or levels of the gene comprising the polynucleotide sequence of SEQ ID NO:1.

Other objects, features, advantages and aspects of the present invention will become apparent to those of skill in the art from the following description. It should be understood, however, that the following description and the specific examples, while indicating preferred embodiments of the invention are given by way of illustration only. Various

changes and modifications within the spirit and scope of the disclosed invention will become readily apparent to those skilled in the art from reading the following description and from reading the other parts of the present disclosure.

SUMMARY OF THE INVENTION

Toward these ends, and others, it is an object of the present invention to provide a method for diagnosing the presence of gynecologic cancers by analyzing for changes in levels of ESBPIII in cells, tissues or bodily fluids compared with levels of ESBPIII in preferably the same cells, tissues, or bodily fluid type of a normal human control, wherein a change in levels of ESBPIII in the patient versus the normal human control is associated with a gynecologic cancer.

Further provided is a method of diagnosing a metastatic gynecologic cancer in a patient which is not known to have metastasized by identifying a human patient suspected of having a gynecologic cancer that has metastasized; analyzing a sample of cells, tissues, or bodily fluid from such patient for ESBPIII; and comparing the ESBPIII levels in such cells, tissues, or bodily fluid with levels of ESBPIII in preferably the same cells, tissues, or bodily fluid type of a normal human control, wherein an increase in ESBPIII levels in the patient versus the normal human control is associated with a gynecologic cancer which has metastasized.

Also provided by the invention is a method of staging gynecologic cancers in a human by identifying a human patient having a gynecologic cancer; analyzing a sample of cells, tissues, or bodily fluid from such patient for ESBPIII; comparing ESBPIII levels in such cells, tissues, or bodily fluid with levels of ESBPIII in preferably the same cells, tissues, or bodily fluid type of a normal human control, wherein an increase in ESBPIII levels in the patient versus the normal human control is associated with a cancer which is progressing and a decrease in the levels of ESBPIII is

associated with a cancer which is regressing or in remission.

Further provided is a method of monitoring gynecologic cancers in a human having such cancer for the onset of metastasis. The method comprises identifying a human patient having such cancer that is not known to have metastasized; periodically analyzing a sample of cells, tissues, or bodily fluid from such patient for ESBPIII; comparing the ESBPIII levels in such cells, tissue, or bodily fluid with levels of ESBPIII in preferably the same cells, tissues, or bodily fluid type of a normal human control, wherein an increase in ESBPIII levels in the patient versus the normal human control is associated with a cancer which has metastasized.

Further provided is a method of monitoring the change in stage of a gynecologic cancer in a human patient by monitoring levels of ESBPIII in the patient. The method comprises identifying a human patient having a gynecologic cancer; periodically analyzing a sample of cells, tissues, or bodily fluid from such patient for ESBPIII; comparing the ESBPIII levels in such cells, tissue, or bodily fluid with levels of ESBPIII in preferably the same cells, tissues, or bodily fluid type of a normal human control sample, wherein an increase in ESBPIII levels in the patient versus the normal human control is associated with a cancer which is progressing and a decrease in the levels of ESBPIII is associated with a cancer which is regressing or in remission.

Further provided are antibodies which specifically bind ESBPIII or fragments of such antibodies which can be used to detect or image localization of ESBPIII in a patient for the purpose of detecting or diagnosing a disease or condition. Such antibodies can be polyclonal, monoclonal, or omniclonal or prepared by molecular biology techniques. The term "antibody", as used herein and throughout the instant specification is also meant to include aptamers and single-stranded oligonucleotides such as those derived from an *in vitro* evolution protocol referred to as SELEX and well known

to those skilled in the art. Antibodies can be labeled with a variety of detectable labels including, but not limited to, radioisotopes and paramagnetic metals. These antibodies or fragments thereof can also be used as therapeutic agents in the treatment of diseases characterized by expression of a ESBPIII. In therapeutic applications, the antibody can be used without or with derivatization to a cytotoxic agent such as a radioisotope, enzyme, toxin, drug or a prodrug.

Other objects, features, advantages and aspects of the present invention will become apparent to those of skill in the art from the following description. It should be understood, however, that the following description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only. Various changes and modifications within the spirit and scope of the disclosed invention will become readily apparent to those skilled in the art from reading the following description and from reading the other parts of the present disclosure.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to diagnostic assays and methods, both quantitative and qualitative for detecting, diagnosing, monitoring, staging and prognosticating cancers by comparing levels of ESBPIII with those of ESBPIII in a normal human control. What is meant by levels of ESBPIII as used herein, means levels of the native protein expressed by the gene comprising the polynucleotide sequence of SEQ ID NO: 1. The protein encoded by this polynucleotide is depicted in SEQ ID NO: 2. In the alternative, what is meant by levels of ESBPIII as used herein, means levels of the native mRNA encoded by the gene comprising the polynucleotide sequence of SEQ ID NO: 1 or levels of the gene comprising the polynucleotide sequence of SEQ ID NO:1. Such levels are preferably measured in at least one of cells, tissues and/or bodily fluids, including determination of normal and abnormal

levels. Thus, for instance, a diagnostic assay in accordance with the invention for diagnosing overexpression of ESBPIII protein compared to normal control bodily fluids, cells, or tissue samples may be used to diagnose the presence of cancers, in particular gynecologic cancers including breast, uterine, ovarian and endometrial cancer.

All the methods of the present invention may optionally include measuring levels of other cancer markers as well as ESBPIII. Other cancer markers, in addition to ESBPIII, useful in the present invention will depend on the cancer being tested and are known to those of skill in the art.

Diagnostic Assays

The present invention provides methods for diagnosing the presence of a gynecologic cancer such as uterine, breast, endometrial or ovarian cancer by analyzing for changes in levels of ESBPIII in cells, tissues or bodily fluids compared with levels of ESBPIII in cells, tissues or bodily fluids of preferably the same type from a normal human control, wherein a change in levels of ESBPIII in the patient versus the normal human control is associated with the presence of a gynecologic cancer.

Without limiting the instant invention, typically, for a quantitative diagnostic assay a positive result indicating the patient being tested has cancer is one in which cells, tissues or bodily fluid levels of a cancer marker, such as ESBPIII, are at least two times higher, and most preferably are at least five times higher, than in preferably the same cells, tissues or bodily fluid of a normal human control.

The present invention also provides a method of diagnosing the onset of metastasis of gynecologic cancers in a patient having a gynecologic cancer which has not yet metastasized. In the method of the present invention, a human cancer patient suspected of having a gynecologic cancer which may have metastasized (but which was not previously known to have metastasized) is identified. This is accomplished by a

variety of means known to those of skill in the art.

In the present invention, determining the presence of ESBPIII levels in cells, tissues or bodily fluid, is particularly useful for discriminating between a gynecologic cancer which has not metastasized and a gynecologic cancer which has metastasized. Existing techniques have difficulty discriminating between gynecologic cancers which have metastasized and gynecologic cancers which have not metastasized. However, proper treatment selection is often dependent upon such knowledge.

In the present invention, the cancer marker level measured in such cells, tissues or bodily fluid is ESBPIII. Measure ESBPIII levels in a human patient are compared with levels of ESBPIII in preferably the same cells, tissue or bodily fluid type of a normal human control. That is, if the cancer marker being observed is ESBPIII in serum, this level is preferably compared with the level of ESBPIII in serum of a normal human control. An increase in the ESBPIII in the patient versus the normal human control is associated with a gynecologic cancer which has metastasized.

Without limiting the instant invention, typically, for a quantitative diagnostic assay a positive result indicating the cancer in the patient being tested or monitored has metastasized is one in which levels of a cancer marker such as ESBPIII in cells, tissues or bodily fluid from the patient are at least two times higher, and most preferably are at least five times higher, than in preferably the same cells, tissues or bodily fluid of a normal human control.

Normal human control as used herein includes a human patient without cancer and/or non cancerous samples from the patient; in the methods for diagnosing or monitoring for metastasis, normal human control may preferably also include samples from a human patient that is determined by reliable methods to have uterine, breast, ovarian or endometrial cancer which has not metastasized.

Staging

The invention also provides a method of staging a gynecologic cancer in a human patient. The method comprises identifying a human patient having such cancer and analyzing cells, tissues or bodily fluid from the patient for ESBPIII. The measured ESBPIII levels in such cells, tissues or bodily fluid from the patient are then compared with levels of ESBPIII in preferably the same cells, tissues or bodily fluid type of a normal human control, wherein an increase in ESBPIII levels in the human patient versus the normal human control is associated with a cancer which is progressing and a decrease in the levels of ESBPIII is associated with a cancer which is regressing or in remission.

Monitoring

Further provided is a method of monitoring gynecologic cancers in a human patient having such cancer for the onset of metastasis. The method comprises identifying a human patient having such cancer that is not known to have metastasized; periodically analyzing cells, tissues or bodily fluid from such human patient for ESBPIII; and comparing the ESBPIII levels in such cells, tissues or bodily fluid with levels of ESBPIII in preferably the same cells, tissues or bodily fluid type of a normal human control, wherein an increase in ESBPIII levels in the human patient versus the normal human control is associated with a cancer which has metastasized.

Further provided by this invention is a method of monitoring the change in stage of a gynecologic cancer in a human patient having such cancer. The method comprises identifying a human patient having such cancer; periodically analyzing cells, tissues or bodily fluid from such human patient for ESBPIII; comparing the ESBPIII levels in such cells, tissues or bodily fluid with levels of ESBPIII in preferably the same cells, tissues or bodily fluid type of a normal human control, wherein an increase in ESBPIII levels

in the human patient versus the normal human control is associated with a cancer which is progressing in stage and a decrease in the levels of ESBPIII is associated with a cancer which is regressing in stage or in remission.

Monitoring patients for onset of metastasis is periodic and preferably done on a quarterly basis. However, this may be more or less frequently depending on the cancer, the particular patient, and the stage of the cancer.

Assay Techniques

Assay techniques that can be used to determine levels of gene expression (including protein levels), such as ESBPIII in the present invention, in a sample derived from a patient are well known to those of skill in the art. Such assay methods include, without limitation, radioimmunoassays, reverse transcriptase PCR (RT-PCR) assays, immunohistochemistry assays, *in situ* hybridization assays, competitive-binding assays, Western Blot analyses, ELISA assays and proteomic approaches: two-dimensional gel electrophoresis (2D electrophoresis) and non-gel based approaches such as mass spectrometry or protein interaction profiling. Among these, ELISAs are frequently preferred to diagnose a gene's expressed protein in biological fluids.

An ELISA assay initially comprises preparing an antibody, if not readily available from a commercial source, specific to ESBPIII, preferably a monoclonal antibody. In addition, a reporter antibody generally is prepared which also binds specifically to ESBPIII. The reporter antibody is attached to a detectable reagent such as radioactive, fluorescent or enzymatic reagent. For example, horseradish peroxidase enzyme or alkaline phosphatase are routinely used as detectable reagents.

To carry out the ELISA, antibody specific to ESBPIII is incubated on a solid support, e.g. a polystyrene dish, that binds the antibody. Any free protein binding sites on the dish are then covered by incubating with a non-specific

protein such as bovine serum albumin. Next, the sample to be analyzed is incubated in the dish, during which time ESBPIII binds to the specific antibody attached to the polystyrene dish. Unbound sample is washed out with buffer. A reporter antibody specifically directed to ESBPIII and linked to a detectable reagent such as horseradish peroxidase is placed in the dish resulting in binding of the reporter antibody to any monoclonal antibody bound to ESBPIII. Unattached reporter antibody is then washed out. Reagents for peroxidase activity, including a colorimetric substrate are then added to the dish. Immobilized peroxidase, linked to ESBPIII antibodies, produces a colored reaction product. The amount of color developed in a given time period is proportional to the amount of ESBPIII protein present in the sample. Quantitative results typically are obtained by reference to a standard curve.

A competition assay can also be employed wherein antibodies specific to ESBPIII are attached to a solid support and labeled ESBPIII and a sample derived from the host are passed over the solid support. The amount of label detected which is attached to the solid support can be correlated to a quantity of ESBPIII in the sample.

Nucleic acid methods can also be used to detect ESBPIII mRNA as a marker for gynecologic cancers such as uterine, breast, endometrial and ovarian cancer. Polymerase chain reaction (PCR) and other nucleic acid methods, such as ligase chain reaction (LCR) and nucleic acid sequence based amplification (NASABA), can be used to detect malignant cells for diagnosis and monitoring of various malignancies. For example, reverse-transcriptase PCR (RT-PCR) is a powerful technique which can be used to detect the presence of a specific mRNA population in a complex mixture of thousands of other mRNA species. In RT-PCR, an mRNA species is first reverse transcribed to complementary DNA (cDNA) with use of the enzyme reverse transcriptase; the cDNA is then amplified

as in a standard PCR reaction. RT-PCR can thus reveal by amplification the presence of a single species of mRNA. Accordingly, if the mRNA is highly specific for the cell that produces it, RT-PCR can be used to identify the presence of a specific type of cell.

Hybridization to clones or oligonucleotides arrayed on a solid support (i.e. gridding) can be used to detect the expression of and quantitate the level of expression of that gene. In this approach, a cDNA encoding the ESBPIII gene is fixed to a substrate. The substrate may be of any suitable type including but not limited to glass, nitrocellulose, nylon or plastic. At least a portion of the DNA encoding the ESBPIII gene is attached to the substrate and then incubated with the analyte, which may be RNA or a complementary DNA (cDNA) copy of the RNA, isolated from the tissue of interest. Hybridization between the substrate bound DNA and the analyte can be detected and quantitated by several means including, but not limited to, radioactive labeling or fluorescence labeling of the analyte or a secondary molecule designed to detect the hybrid. Quantitation of the level of gene expression can be done by comparison of the intensity of the signal from the analyte compared with that determined from known standards. The standards can be obtained by *in vitro* transcription of the target gene, quantitating the yield, and then using that material to generate a standard curve.

Of the proteomic approaches, 2D electrophoresis is a technique well known to those in the art. Isolation of individual proteins from a sample such as serum is accomplished using sequential separation of proteins by different characteristics usually on polyacrylamide gels. First, proteins are separated by size using an electric current. The current acts uniformly on all proteins, so smaller proteins move farther on the gel than larger proteins. The second dimension applies a current perpendicular to the first and separates proteins not on the basis of size but on

the specific electric charge carried by each protein. Since no two proteins with different sequences are identical on the basis of both size and charge, the result of a 2D separation is a square gel in which each protein occupies a unique spot. Analysis of the spots with chemical or antibody probes, or subsequent protein microsequencing can reveal the relative abundance of a given protein and the identity of the proteins in the sample.

The above tests can be carried out on samples derived from a variety of cells, bodily fluids and/or tissue extracts (homogenates or solubilized tissue) obtained from a patient including tissue biopsy and autopsy material. Bodily fluids useful in the present invention include blood, urine, saliva or any other bodily secretion or derivative thereof. Blood can include whole blood, plasma, serum or any derivative of blood.

In Vivo Antibody Use

Antibodies against ESBPIII can also be used *in vivo* in patients suspected of suffering from gynecologic cancers such as ovarian, breast, endometrial and uterine cancer. Specifically, antibodies against a ESBPIII can be injected into a patient suspected of having a gynecologic cancer for diagnostic and/or therapeutic purposes. The use of antibodies for *in vivo* diagnosis is well known in the art. For example, antibody-chelators labeled with Indium-111 have been described for use in the radioimmunoscintographic imaging of carcinoembryonic antigen expressing tumors (Sumerdon et al. Nucl. Med. Biol. 1990 17:247-254). In particular, these antibody-chelators have been used in detecting tumors in patients suspected of having recurrent colorectal cancer (Griffin et al. J. Clin. Onc. 1991 9:631-640). Antibodies with paramagnetic ions as labels for use in magnetic resonance imaging have also been described (Lauffer, R.B. Magnetic

Resonance in Medicine 1991 22:339-342). Antibodies directed against ESBPIII can be used in a similar manner. Labeled antibodies against ESBPIII can be injected into patients suspected of having a gynecologic cancer for the purpose of diagnosing or staging of the disease status of the patient. The label used will be selected in accordance with the imaging modality to be used. For example, radioactive labels such as Indium-111, Technetium-99m or Iodine-131 can be used for planar scans or single photon emission computed tomography (SPECT). Positron emitting labels such as Fluorine-19 can be used in positron emission tomography. Paramagnetic ions such as Gadlinium (III) or Manganese (II) can be used in magnetic resonance imaging (MRI). Localization of the label permits determination of the spread of the cancer. The amount of label within an organ or tissue also allows determination of the presence or absence of cancer in that organ or tissue.

For patients diagnosed with a gynecologic cancer, injection of an antibody against ESBPIII can also have a therapeutic benefit. The antibody may exert its therapeutic effect alone. Alternatively, the antibody is conjugated to a cytotoxic agent such as a drug, toxin or radionuclide to enhance its therapeutic effect. Drug monoclonal antibodies have been described in the art for example by Garnett and Baldwin, Cancer Research 1986 46:2407-2412. The use of toxins conjugated to monoclonal antibodies for the therapy of various cancers has also been described by Pastan et al. Cell 1986 47:641-648. Yttrium-90 labeled monoclonal antibodies have been described for maximization of dose delivered to the tumor while limiting toxicity to normal tissues (Goodwin and Meares Cancer Supplement 1997 80:2675-2680). Other cytotoxic radionuclides including, but not limited to Copper-67, Iodine-131 and Rhenium-186 can also be used for labeling of antibodies against ESBPIII.

Antibodies which can be used in these *in vivo* methods include both polyclonal, monoclonal or omniclonal antibodies and antibodies prepared via molecular biology techniques. Antibody fragments and aptamers and single-stranded oligonucleotides such as those derived from an *in vitro* evolution protocol referred to as SELEX and well known to those skilled in the art can also be used.

EXAMPLES

The present invention is further described by the following examples. The examples are provided solely to illustrate the invention by reference to specific embodiments. These exemplifications, while illustrating certain specific aspects of the invention, do not portray the limitations or circumscribe the scope of the disclosed invention.

The examples are carried out using standard techniques, which are well known and routine to those of skill in the art, except where otherwise described in detail. Routine molecular biology techniques of the following example can be carried out as described in standard laboratory manuals, such as Sambrook et al., MOLECULAR CLONING: A LABORATORY MANUAL, 2nd Ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989).

Real-Time quantitative PCR with fluorescent Taqman probes is a quantitation detection system utilizing the 5'- 3' nuclease activity of Taq DNA polymerase. The method uses an internal fluorescent oligonucleotide probe (Taqman) labeled with a 5' reporter dye and a downstream, 3' quencher dye. During PCR, the 5'-3' nuclease activity of Taq DNA polymerase releases the reporter, whose fluorescence can then be detected by the laser detector of the Model 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA, USA).

Amplification of an endogenous control is used to

standardize the amount of sample RNA added to the reaction and normalize for Reverse Transcriptase (RT) efficiency. Either cyclophilin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or 18S ribosomal RNA (rRNA) is used as this endogenous control. To calculate relative quantitation between all the samples studied, the target RNA levels for one sample were used as the basis for comparative results (calibrator). Quantitation relative to the "calibrator" is obtained using the standard curve method or the comparative method (User Bulletin #2: ABI PRISM 7700 Sequence Detection System).

To evaluate the tissue distribution, and the level of ESBPIII in normal and tumor tissue, total RNA was extracted from normal tissues, tumor tissues, and from tumors and the corresponding matched normal tissues. Subsequently, first strand cDNA was prepared with reverse transcriptase and the polymerase chain reaction was done using primers and Taqman probe specific to ESBPIII. The results are analyzed using the ABI PRISM 7700 Sequence Detector. The absolute numbers are relative levels of expression of ESBPIII compared to the calibrator tissue.

The absolute numbers depicted in Table 1 are relative levels of expression of ESBPIII in 12 normal different tissues. All the values are compared to normal mammary gland (calibrator). These RNA samples are commercially available pools, originated by pooling samples of a particular tissue from different individuals.

Table 1: Relative Levels of ESBPIII Expression in Pooled Samples

Tissue	NORMAL
Brain	0
Heart	0
Kidney	0
Liver	0
Lung	0
Breast	1
Prostate	0
Small Intestine	0
Spleen	0
Testis	1
Thymus	0
Uterus	59

The relative levels of expression in Table 1 show that the highest level of expression of ESBPIII mRNA is in uterus (59), with expression also in mammary gland (1), and testis (1). These results establish that ESBPIII mRNA expression is highly specific for uterus and breast in gynecologic tissues, and testis for male tissues.

The absolute numbers in Table 1 were obtained analyzing pools of samples of a particular tissue from different individuals. They can not be compared to the absolute numbers originated from RNA obtained from tissue samples of a single individual in Table 2.

The absolute numbers depicted in Table 2 are relative levels of expression of ESBPIII in 55 pairs of matching samples, ovarian cancer samples from 5 different individuals, and normal ovarian samples from 5 different individuals. All the values are compared to normal mammary gland (calibrator). A matching pair is formed by mRNA from the cancer sample for a particular tissue and mRNA from the normal adjacent sample for that same tissue from the same individual.

Table 2: Relative Levels of ESBPIII Expression in Pooled Samples

Sample ID	Tissue	Cancer Tissue	Normal Adjacent Tissue	Normal Tissue
End10479	Endometrium 1	0	2	
End8911	Endometrium 2	1413	274	
Endo12XA	Endometrium 3	19	9	
Endo28XA	Endometrium 4	1680	174	
Endo3AX	Endometrium 5	4	4	
Endo5XA	Endometrium 6	97	454	
Endo65RA	Endometrium 7	192	12	
Endo8XA	Endometrium 8	1	485	
End8963	Endometrium 9	1413	4	
End4XA	Endometrium 10	1	0	
End68X	Endometrium 11	984	1714	
Bld32XK	Bladder 1	0	0	
Bld46XK	Bladder 2	0	0	
ClnAS45	Colon 1	0	0	
ClnRC01	Colon 2	1	3	
ClnB34	Colon 3	0	0	
CvxKS52	Cervix 1	0	0	
CvxNKS18	Cervix 2	0	0	
CvxNKs80	Cervix 3	0	0	
Kid107XD	Kidney 1	0	1	
Kid106XD	Kidney 2	2	1	
Liv15XA	Liver 1	0	0	
Liv94XA	Liver 2	0	0	
Lng60XL	Lung 1	0	1	
LngC20X	Lung 2	0	0	

Mam47XP	Breast 1	1	0	
Mam82XI	Breast 2	0	1	
MamA06X	Breast 3	1	0	
MamB011X	Breast 4	0	0	
Mam59X	Breast 5	0	0	
Mam162X	Breast 6	0.03	0.14	
Mam19DN	Breast 7	133.09	2.04	
Mam220	Breast 8	0.48	0.27	
Mam76DN	Breast 9	0.51	10.46	
MamS079	Breast 10	0.07	0.12	
MamS127	Breast 11	0.52	0.44	
MamS621	Breast 12	0.07	0.39	
Pan71XL	Pancreas 1	0	0	
Pan82XP	Pancreas 2	0	0	
Pro18XB	Prostate 1	0.0	0.3	
Pro20XB	Prostate 2	3.3	1.3	
Pro69XB	Prostate 3	0	0.3	
Pro90XB	Prostate 4	0	0	
Pro65XB	Prostate 5	0	3	
SmInt21XA	Small Intestine 1	0	0	
SmInH89	Small Intestine 2	0	0	
StoAC44	Stomach 1	0	4	
StoAC99	Stomach 2	2	5	
Tst39X	Testis 1	0	0	
Utr135XO	Uterus 1	19	14	
Utr141XO	Uterus 2	25	3	
Utr85XU	Uterus 3	1148	680	
Utr23XU	Uterus 4	1013	60	

Ovr103X	Ovary 1	111	0	
Ovr130X	Ovary 2	0	3	
Ovr1005	Ovary Cancer 1	28		
Ovr1040	Ovary Cancer 2	60		
Ovr1157	Ovary Cancer 3	109		
Ovr63A	Ovary Cancer 4	0		
Ovr1028	Ovary Cancer 5	0		
Ovr230A	Ovary Normal 1			0
Ovr32RA	Ovary Normal 2			0
Ovr40G	Ovary Normal 3			0
Ovr35GA	Ovary Normal 4			0
Ovr9RA	Ovary Normal 5			0

0= Negative

In the analysis of matching samples, the higher levels of expression for ESBPIII were in uterus, endometrium, ovary, and breast. This pattern shows a high degree of specificity for female gynecologic tissues, especially for endometrium, uterus, and ovary. These results confirmed the tissue specificity results obtained with the panel of normal pooled samples (Table 1) for uterus and breast.

Furthermore, the levels of mRNA expression in cancer samples and the isogenic normal adjacent tissue from the same individual were compared. This comparison provides an indication of specificity for the cancer stage (e.g. higher levels of mRNA expression in the cancer sample compared to the normal adjacent). Table 2 shows overexpression of ESBPIII in 6 primary endometrial cancer tissues compared with their respective normal adjacent (endometrium samples #2, 3, 4, 7, 9 and 10). There was overexpression in the cancer tissue for 54.54% of the endometrial matching samples tested (total of 11

endometrium matching samples).

ESBPIII is differentially expressed in the four matching samples for uterine cancer. All four samples analyzed showed overexpression in cancer. Of twelve breast cancer matching samples analyzed, five showed underexpression of ESBPIII (#2, 6, 9, 10 and 12)in cancer, whereas five had higher levels of ESBPIII in cancer compared to the normal adjacent tissue (#1, 3, 7, 8, and 11). Two of the breast matching samples do not show expression of ESBPIII mRNA.

ESBPIII is differentially expressed in the two matching samples for ovarian cancer. Sample #1 shows upregulation for the mRNA of ESBPIII in cancer, whereas sample #2 shows overexpression in the normal adjacent tissue. In addition to the two matching samples, ten additional samples for ovary were analyzed including five cancer samples and five normal ovary tissue samples from different individuals. Expression of ESBPIII mRNA was observed in three of the cancer samples (#1, 2, and 3). The median expression in the ovary cancer samples was 28.1, whereas expression in the normal ovary samples was 0.

Altogether, the high level of tissue specificity for gynecological tissues, plus the mRNA differential expression in several of the primary uterus, endometrial, breast, and ovarian matching samples tested is indicative of ESBPIII being a diagnostic marker for gynecologic cancers including uterine, endometrial, breast, and ovarian cancer.